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Environmental and nutrient controls of marine nitrogen fixation

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ABSTRACT

Biological dinitrogen (N_2) fixation by diazotrophic cyanobacteria has great biogeochemical implications in nitrogen (N) cycling in the ocean as this process represents the major source of new N input to the oceans, thereby controlling the marine primary productivity. Numerous factors can affect the extent of N_2 fixation. A better understanding of the major environmental and nutrient factors governing this process is highly required. Iron (Fe) and/or phosphorus (P) are thought to be limiting factors in most oceanic regions. Special attention has been given in the present study to evaluate the effects of mineral dust deposition which is believed to stimulate N_2 fixation as it increases the availability of both Fe and P. Through three laboratory bioassays (+Fe, +P, +Dust) via incubation experiments performed on *Trichodesmium* IMS101, we found that each addition of Fe, P or desert dust could stimulate the growth and N_2 fixation of *Trichodesmium* IMS101. Several adaptive nutrient utilization strategies were observed, such as a Fe luxury uptake mechanism, a P-sparing effect and colony formation. In addition, during a field study using natural phytoplankton assemblages from the temperate Northeast Atlantic Ocean the critical role of dissolved Fe (DFe) was again highlighted by the remarkably enhanced N_2 fixation rate observed after the addition of DFe under low temperature and P-depleted conditions. At the time of our study no *Trichodesmium* filaments were found in the studied region, the diazotrophic community was dominated by unicellular cyanobacteria symbiont (prymnesiophyte-UCYN-A1) and heterotrophic diazotrophs, therefore demonstrating that DFe could as well be the ultimate factor limiting N_2 fixation of these smaller diazotrophs. Recently, the effects of ongoing climate change (ocean warming and acidification) on N_2 fixation drew much attention, but various studies led to controversial conclusions. Semi-continuous dilution growth experiments were conducted on *Trichodesmium* IMS101 under present-day and future high pCO_2 (400 and 800 μatm, respectively) and warming seawater (24 and 28 °C) conditions. The results indicate that higher pCO_2 and therefore ocean acidification may be beneficial for *Trichodesmium* growth and N_2 fixation. However, our observations suggest that Fe or P limitation in oligotrophic seawaters may offset the stimulation induced on *Trichodesmium* IMS101 resulting from ocean acidification. In contrast, ocean warming may not play an important role in *Trichodesmium* growth and N_2 fixation with a 4 °C increase from 24 °C to 28 °C. Nevertheless, ocean warming is predicted to cause a shift in the geographical distribution of *Trichodesmium* species toward higher latitudes, extending its niche to subtropical ocean regions and potentially reducing its coverage in tropical ocean basins.

1. Introduction

Diazotrophic cyanobacteria play a crucial role in marine ecosystem functioning and biogeochemical cycling of nitrogen (N) and carbon (C). Owing to their ability to convert atmospheric N_2 into ammonia (NH_3) by nitrogenase activity they provide the major source of new N to large parts of the oligotrophic oceans and control the N budget (Karl et al., 2002). Furthermore, as N is the limiting nutrient in vast regions of the ocean, this new N can fuel primary productivity and the associated mechanisms (carbon bio-pump) for atmospheric carbon dioxide (CO_2) sequestration in the deep ocean.

Trichodesmium spp, a marine colony-forming filamentous N_2 fixing cyanobacterium, is a major and abundant N_2 fixer and has been extensively studied (e.g., Capone et al., 2005; Breitbarth et al., 2007; Hutchins et al., 2007; Fernandez et al., 2010). *Trichodesmium* spp. is estimated to account for an input of about 80–110 Tg N per year into the ocean, which is more than half of the global marine N_2 fixation input (between 100 and 200 Tg N per year) (Capone et al., 2005). *Trichodesmium* has long been considered as a dominant contributor to oceanic N_2 fixation and are found essentially in the oligotrophic tropical and subtropical oceans (Capone et al., 2005; Mahaffey et al., 2005). Nevertheless, there exists nowadays plenty of evidence revealing

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that the role of smaller diazotrophs (i.e., unicellular N_2-fixing cyanobacteria and heterotrophic diazotrophs) has been severely underestimated and that their N_2 fixation activity probably equals or exceeds that of *Trichodesmium* (Zehr, 2011). A variety of field studies has shown that these unicellular diazotrophic cyanobacteria (UCYN) and non-cyanobacterial diazotrophs are highly abundant and may account for substantial amounts of N introduction in many oligotrophic regions (Montoya et al., 2004; Moisander et al., 2010). These cyanobacteria include Group UCYN-A, presently uncultivated under laboratory conditions, Group UCYN-B (*Crocosphaera watsonii*) and UCYN-C, already investigated in laboratory experiments. Recent research suggests that UCYN-A cells among which three clades have been identified (UCYN-A1, UCYN-A2 and UCYN-A3; Thompson et al., 2014) live in symbiotic association with unicellular photosynthetic eukaryotes prymnesiophyte (Thompson et al., 2012; Krupke et al., 2014, 2015; Cabello et al., 2016).

Numerous factors (CO_2 level, temperature, light, oxygen, turbulence, salinity, inorganic nutrients and trace metals) appear to affect the extent of N_2 fixation (Karl et al., 2002). In view of the importance of N_2 fixation in the global C and N cycles, a better understanding of the major factors controlling N_2 fixation is required. It has been widely acknowledged that Fe is the key controlling factor of marine N_2 fixation, not only because of the high Fe content of nitrogenase and the photosynthetic enzymes complex, but also because of the very low Fe concentrations in oceanic surface seawaters (Berman-Frank et al., 2007). Phosphorus (P) is a fundamental element required for bacterial and phytoplankton growth and since its availability is limited in some oceanic regions, its supply is also commonly believed to constrain N_2 fixation (Sanudo-Wilhelmy et al., 2001). Thus, a great deal of in-vitro and field investigations studying the nutritional requirements of *Trichodesmium* have focused on the effects of Fe and P limitations. These studies suggest that oceanic N_2 fixation can be limited by either Fe (Wu et al., 2000; Kustka et al., 2003a; Moore et al. 2009), P (Sanudo-Wilhelmy et al., 2001; Moutin et al., 2005; Sohm et al., 2008) or co-limited by both Fe and P (Mills et al., 2004). In addition, it has been hypothesized that access to nutrients delivered by desorption from settled atmospheric dust particles affects diazotrophic activity in the ocean (Mills et al., 2004; Ridame et al., 2011; Langlois et al., 2012).

Meanwhile, oceanic systems are undergoing continuous modifications at varying rates and magnitudes as a result of changing climate. Since the onset of the industrial revolution about one-third of the anthropogenic CO_2 released into the atmosphere has been absorbed by the ocean via biological and physical-chemical processes. Dissolution of this greenhouse gas has led to increased partial pressures of CO_2 (pCO_2) and reduced pH of surface seawater. This ocean acidification has been shown to have various consequences for marine phytoplankton. As for the biological responses of diazotrophs to increased CO_2 concentrations, studies to date mostly focused on *Trichodesmium* IMS101. Most investigations indicate that elevated pCO_2 not only significantly increases growth rates and biomass production but also stimulates photosynthesis and N_2 fixation of *Trichodesmium* IMS101 cultures (Barcelos e Ramos et al., 2007; Hutchins et al., 2007; Levitan et al., 2007, 2010; Kranz et al., 2009, 2010; Hutchins et al., 2015; Walworth et al., 2016a, 2016b). Sea-surface temperature is increasing globally along with pCO_2 and the distribution of *Trichodesmium* is thought to be limited geographically by temperature. Elevated seawater temperature may significantly extend the geographic distribution of *Trichodesmium* to higher latitudes (Breitbarth et al., 2007). However, with continued warming N_2 fixation by *Trichodesmium* could decrease in some tropical regions in case warming exceeds the optimal temperatures for growth (Thomas et al., 2012). Furthermore, ocean warming would enhance water stratification and thereby lower nutrient input from deep waters into the upper mixed layer, leading to a decreased N availability in the surface ocean. Therefore, N_2 fixation may play a more important role in the marine N cycle as ocean acidification and seawater warming intensify.

It is critically important to understand how diazotrophic cyanobacteria respond to nutrient limitation, temperature and pCO_2 in order to predict the impact of environmental change on N_2 fixation, and how this might affect global biogeochemical cycles, food web dynamics and overall productivity of the open oceans. We have carried out three bioassay (+Fe, +P, +Dust) incubation experiments to evaluate the effects of nutrient and dust additions on *Trichodesmium* IMS101's growth and N_2 fixation. In addition, through a field study conducted in the Bay of Biscay and along the Iberian Margin in May 2014, we investigated the impact of DFe on N_2 fixation via field incubation experiments using natural phytoplankton assemblages. Finally, semi-continuous dilution growth experiments were conducted on *Trichodesmium* IMS101 under present-day and future high pCO_2 (400 and 800 µatm, respectively) as well as warming seawater (24 and 28 °C) conditions, in order to evaluate the influence of ocean acidification and warming on diazotroph growth and N_2 fixation activity.

2. Material and methods

2.1. Cultures and media preparation

The *Trichodesmium erythraeum* IMS101 strain was obtained from the National Centre for Marine Algae and Microbiota (NCMA, Bigelow Laboratory for Marine Sciences, East Boothbay Harbor, Maine 04544, USA) and isolated from coastal waters, North Carolina USA. Monospecific stock cultures of *Trichodesmium* IMS101 were maintained in N-free, P- and Fe-replete artificial modified YBCII medium (Chen et al., 1996). The pH of the medium was adjusted at 8.15–8.2. The dissolved phosphate (PO_4^{3-}) and Fe concentrations in the medium were 5 µM and 0.3 µM, respectively. The stock cultures of *Trichodesmium* IMS101 were maintained in 750 mL sterile polystyrene culture flasks at 24 °C, with 150 µmol photons m^{-2} s^{-1} incident irradiance and a 14–10 h light – dark cycle. Monospecific stock cultures were grown and kept under exponential growth phase conditions through successive dilutions with fresh YBC II medium.

2.2. Experimental setup

2.2.1. Nutrient addition bioassay experiments

Three nutrient addition (+Fe, +P, +Dust) bioassay incubation experiments were designed to investigate the effects of P, Fe and dust (Kubuqi desert dust) additions on growth and N_2 fixation activity of *Trichodesmium* IMS101. Nutrient limiting conditions were achieved by gradual dilution with YCB II medium depleted in the nutrient under investigation. For example, for Fe addition experiments, once the strain reached the mid-exponential phase, it was diluted with Fe-depleted and P-replete YCB II medium. After several dilutions, the culture was kept under Fe-limiting conditions. For dust addition, the culture was manipulated under Fe- and P-co-limiting conditions. Once the nutrient concentrations were low enough (for instance, DFe < 10 nM, PO_4^{3-} < 100 nM), meeting our requirements, cultures were transferred into 1 L Nalgene polycarbonate bottles and kept at an incident irradiance of 150 µmol photons m^{-2} s^{-1} with a 14–10 h light – dark cycle at 24 °C for 24 h. A 24 h incubation period ensures that the N_2 fixation measurement covers a full daily light cycle.

To measure N_2 fixation rates the improved $^{15}N_2$ tracer method with the addition of $^{15}N_2$-enriched seawater was used instead of the gas bubble method (Mohr et al., 2010; Großkopf et al., 2012). Each 1 L incubation bottle was filled with culture medium, but leaving a 100 mL headspace. Then nutrients were added to reach three different concentration levels for each nutrient addition experiment which was run in duplicate (Table 1). For a 1 L incubation, 60 mL $^{15}N_2$ (99% ^{15}N atom, Eurisotop)-enriched seawater as added to each bottle which was then spiked with 0.7 mL of 0.2 M $NaH^{13}CO_3$ (99% ^{13}C atom, Eurisotop) to determine the assimilation of dissolved inorganic carbon (DIC). Finally, the bottles were topped up with the culture medium leaving no headspace or bubbles, closed well with Teflon-coated butyl rubber septum caps, and placed in the incubator for 24 h. In parallel, a "control" bottle

Table 1
Different nutrient addition levels for +Fe, +P, +Dust bioassay experiments.

Levels	Nutrients		
	+Fe (nM)	+P (nM)	+Dust (mg L^{-1})
1	50	50	25
2	100	100	50
3	200	200	100

without nutrient addition and a "natural" bottle without the addition of nutrients nor isotopic tracers were also prepared and incubated similarly.

Before and after incubation, samples were taken for analyses of fluorescence, cell abundance, Chlorophyll-a (Chl-a), nitrate (NO_3^-), PO_4^{3-}, DFe, particulate organic carbon (POC) and particulate nitrogen (PN) contents and their isotopic compositions. After incubation we also sampled aliquots from the incubated medium into gas tight exetainers to assess the ^{13}C and ^{15}N enrichment of DIC ($\delta^{13}C$-DIC) and dissolved N_2 substrate ($\delta^{15}N$-N_2).

2.2.2. Field experiments

Field incubation experiments using natural phytoplankton assemblages were conducted in the temperate NE Atlantic (Belgica 2014/14 cruise, 21–30 May 2014). This expedition covered six different stations along a North-South section (around 10 °W) located in the Bay of Biscay (Stations 3–7, 46.38°N–43.96°N) and along the Iberian Margin (Stations 9–13, 42.23°N–38.47°N). Sampling stations are shown in Fig. 1.

Seawater samples for biogeochemical measurements, such as NO_3^-, PO_4^{3-} and dissolved Silicate (DSi), Chl-a, POC and PN, were collected using a Sea-Bird SCTD SBE9plus system equipped with 12 of 10 L Niskin bottles and mounted with a conductivity-temperature-depth (CTD) sensor. The vertical light profiles were measured with another Sea-Bird SCTD SBE19plus system equipped with a photosynthetically active radiance (PAR) sensor. To study the role of Fe on oceanic N_2 fixation, natural seawater samples were collected at near-surface level (5 m, 54% incident solar irradiance) for duplicate incubation experiments to assess N_2 fixation rates with DFe amendments. On board, we applied the same improved $^{15}N_2$ tracer method with the addition of $^{15}N_2$-enriched seawater to measure N_2 fixation rates and followed the same procedure used in the nutrient addition bioassay incubation experiments. The difference was that seawater samples were collected into 4.5 L polycarbonate bottles, amended with 250 mL $^{15}N_2$-enriched low-nutrient seawater and spiked with 100 nM dissolved Fe^{3+}. The water samples were then incubated in the on-deck incubators, wrapped with blue film sheets to obtain the corresponding level of light transmission and flushed continuously with surface seawater to maintain at in-situ temperature. Finally, N_2 fixation rates were calculated as described in Montoya et al. (1996). At these six stations seawater samples were also collected in duplicate at depths corresponding to 54%, 13%, 3% and 0.2% of incident solar irradiance to determine in-situ community N_2 fixation activity (without Fe amendments). All incubation experiments started at around 9:00 in the morning and lasted for 24 h. Details about the experimental protocol and the results of these on board incubations are presented in Fonseca-Batista et al. (2018). In-situ diazotrophic activities determined at surface level without DFe could then be compared to those measured in Fe-enriched incubations.

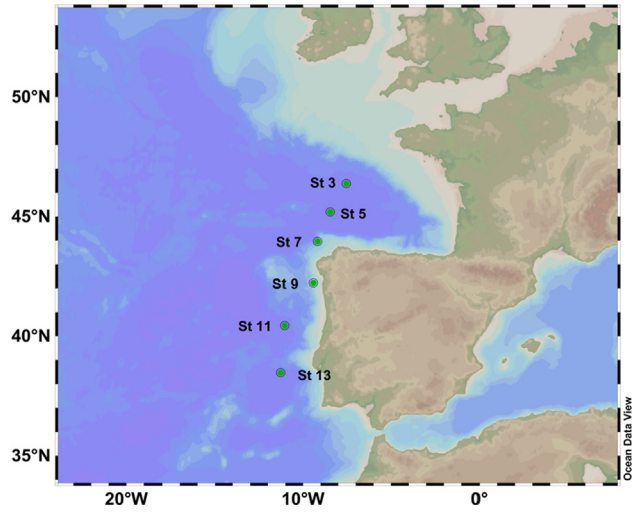

Fig. 1. Sampling stations in the Bay of Biscay and along the Iberian Margin during the Belgica Cruise in May 2014.

2.2.3. Semi-continuous dilution growth experiments

Semi-continuous dilution culturing methods were applied because they allow to better assess the effects of CO_2 and temperature under acclimated and steady-state growth conditions (Fu et al., 2014; Hutchins et al, 2015). Culture experiments on *Trichodesmium* IMS101 were performed at temperatures of 24 °C and 28 °C and at pCO_2 levels of 400 µatm and 800 µatm, corresponding to present-day and near-future conditions. Overall, four pCO_2 and temperature treatments were run: Present-day (400 µatm CO_2 and 24 °C), High Temperature (400 µatm CO_2 and 28 °C), High pCO_2 (800 µatm CO_2 and 24 °C) and High Temperature + High pCO_2 (800 µatm CO_2 and 28 °C). The 400 µatm CO_2 and 24 °C treatment was considered as our control treatment since its settings fit the current ambient atmospheric pCO_2 in oceanic regions and the range of temperatures at which *Trichodesmium* spp. thrive (20–30 °C) (Bates, 2007; Monteiro et al., 2010).

For each targeted combination of pCO_2 and temperature treatment a 4.5 L Nalgene polycarbonate bottle containing the strain and medium was placed in a temperature-controlled incubator at constant incident photon flux density (150 µmol m^{-2} s^{-1}) with a 14–10 h light – dark cycle. The pCO_2 in the cultures was maintained by continuous gentle bubbling of CO_2-free ambient air flow mixed with a CO_2 and O_2 mixture containing 5% CO_2 during the entire acclimation stage. During the acclimation, fluorescence and cell density were measured every 1–2 days as real-time biomass indicators and were used to estimate the specific growth rate (µ) of *Trichodesmium* IMS101. The cell numbers were kept at the lower end of the exponential growth phase (approximately 50,000 cells mL^{-1}) through continuous dilution. Steady-state growth status was considered to have been reached when no significant difference in cell-specific growth rates for at least 3 consecutive transfers was observed. Once steady-state growth was achieved and the cultures were considered to be fully acclimated to target pCO_2 and temperature levels, cultures were then transferred during the exponential growth phase into 1 L Nalgene polycarbonate bottles, using three replicates for each treatment to conduct 24 h incubation experiments. To measure N_2 fixation rates, we followed the same protocol used in the nutrient addition bioassay experiments described in the previous section. Samples were taken for the measurements of pH, alkalinity, pCO_2, fluorescence, cell abundance, Chl-a, NO_3^-, PO_4^{3-}, DFe, POC and PN concentrations and their isotopic compositions, and $\delta^{13}C$-DIC and $\delta^{15}N$-N_2.

2.2.4. Analytical methods

Fluorescence was determined with a Turner fluorometer-turbidimeter and was used as an indicator for *Trichodesmium* growth. Cell abundance was counted on a glass slide under a light microscope (with x100 magnification). Specific growth rates (µ) were calculated as the slope of a linear regression of the natural logarithm of cell concentration against time over the exponential growth phase. Chl-a concentrations were measured following the fluorimetric method of Yentsch and Menzel (1963). Nitrate was analysed colorimetrically with a Skalar Autoanalyzer system (Grasshoff et al., 1983). Phosphate concentration was manually determined by spectrophotometry according to the

molybdate and ascorbic acid method (Grasshoff et al., 1983). DFe concentrations were spectrophotometrically determined at 562 nm, following the ferrozine methods adapted from Viollier et al. (2000). The detection limits associated to NO_3^-, PO_4^{3-} and DFe were 200 nM, 40 nM and 10 nM, respectively. It is important to note that the Ferrozine methods are mostly suitable for determining high Fe concentrations as used in our laboratory culture experiments. POC and PN concentrations and their corresponding isotopic compositions were measured via Elemental Analyser - Isotope Ratio Mass Spectrometry (EA-IRMS, Delta V, Thermo). δ^{13}C-DIC and δ^{15}N-N_2 were determined using an automatic gas sampling system (GasPrep by Nu Instruments), coupled to an IRMS (Perspective, Nu Instruments).

N_2 fixation rates were calculated based on the final isotopic composition of ^{15}N present in PN from the enriched and "Natural" incubations, and the natural abundance of ^{15}N in the dissolved inorganic N_2 pool as described in Montoya et al. (1996) and using the following Eq. (1):

$$N_2 \text{ fixation rate} = \frac{A^{PN}_{Sample} - A^{PN}_{Natural}}{A_{N_2} - A^{PN}_{Natural}} * \frac{[PN]}{\Delta t} \quad (1)$$

where A^{PN}_{Sample} corresponds to ^{15}N atom% of PN samples from incubations to which $^{15}N_2$-enriched seawater was added, $A^{PN}_{Natural}$ denotes those from the "Nature" incubations which were carried out simultaneously without $^{15}N_2$-enriched seawater addition, and A_{N_2} presents the ^{15}N atom% of the dissolved N_2 pool. [PN] (μM) is the final PN concentration in experimental incubations, and Δt (d) is the duration of incubation. Carbon fixation rates were calculated the same way as described for N_2 fixation rates.

2.2.5. Statistical treatment of data

The effects of Fe, P and dust additions on the growth, nutrient uptake mechanisms, and N_2 and C fixation of *Trichodesmium* were evaluated by one-way analysis of variance (ANOVA) with post hoc Tukey test of multiple comparisons. Two-way ANOVA was used to identify whether the different pCO_2 (400 μatm and 800 μatm) and temperature (24 °C and 28 °C) treatments had significant impact on the growth rate and N_2 fixation activity of *Trichodesmium* IMS101. All statistical treatments of data were performed using the SPSS 19.0 software. The confidence level for all analyses was set at 95%.

3. Results

3.1. Nutrient addition bioassay experiments

3.1.1. Fe addition bioassay experiments

For the Fe addition experiments the initial DFe and PO_4^{3-} concentrations were 9 ± 0 nM and 0.63 ± 0.15 μM, respectively. The three different Fe additions applied were as follows: Fe 1 with 50 nM, Fe 2 with 100 nM and Fe 3 with 200 nM (see Table 1).

Iron addition generally stimulated the growth of *Trichodesmium* (one-way ANOVA, $p < 0.05$, Fig. 2a). Growth rates significantly increased with Fe additions (one-way ANOVA, $p < 0.05$, Fig. 2a). The net DFe uptake rates were estimated from the difference in concentrations between the end and the start of the incubations (Fig. 2b). The control showed no measurable DFe consumption during the 24 h incubation (Fig. 2b). In contrast, DFe uptake rates were significantly stimulated for the three Fe addition treatments (one-way ANOVA, $p < 0.05$, Fig. 2b). The mean DFe uptake rates for the 200 nM Fe addition were 2-fold and 5-fold higher than those for the 100 and 50 nM Fe addition treatments, respectively (Fig. 2b). N_2 fixation of *Trichodesmium* was stimulated with Fe additions, although the effect was statistically significant relative to the control only for the 200 nM Fe addition (one-way ANOVA, $p < 0.05$, Fig. 2c). Mean values of N_2 fixation rates with 50, 100, 200 nM Fe additions were 12%, 51% and 73% higher than that of control, respectively (Fig. 2c). Similarly, C fixation rates of *Trichodesmium* generally increased in response to elevated DFe concentrations, however, the effect was significant only for the 200 nM Fe addition (one-way ANOVA, $p < 0.05$, Fig. 2d).

3.1.2. P Addition bioassay experiments

For the P addition experiments, the initial PO_4^{3-} concentration was < 40 nM and DFe was 110 ± 10 nM. There were three different P additions: P 1 with 50 nM, P 2 with 100 nM and P 3 with 200 nM (see Table 1).

Phosphate addition typically had a positive impact on the growth of *Trichodesmium* (one-way ANOVA, Fig. 3a). The P uptake rates were estimated from the difference in concentrations at the start and the end of the incubations (Fig. 3b). While for the control there was no measurable P consumption during 24 h incubation (Fig. 3b), the uptake rates were significantly enhanced for the three P addition treatments (one-way ANOVA, $p < 0.05$, Fig. 3b). Though N_2 fixation rates of *Trichodesmium* were stimulated by P addition, the effect was not statistically significant for the 50 nM P addition relative to the control (one-way ANOVA, $p < 0.05$, Fig. 3c). The carbon fixation rates of *Trichodesmium* increased significantly in response to elevated PO_4^{3-} concentrations (one-way ANOVA, $p < 0.05$, Fig. 3d).

3.1.3. Dust addition bioassay experiments

For the dust addition bioassay experiments the initial DFe and PO_4^{3-} concentrations were 5 ± 0 nM and 90 ± 0 nM, respectively. There were three different dust additions: Dust 1 with 25 mg L^{-1}, Dust 2 with 50 mg L^{-1} and Dust 3 with 100 mg L^{-1} (see Table 1).

Dust addition stimulated the growth of *Trichodesmium* (one-way ANOVA, $p < 0.05$, Fig. 4a). Considerable amounts of PO_4^{3-} (0.14–0.58 μM) and DFe (50–850 nM) were released from the dust into the culture medium during 24 h and the concentrations of PO_4^{3-} and DFe increased with increasing dust loads. Due to the variable dissolution of DFe with time and the relatively low precision of the Ferrozine technique, DFe uptake rates were poorly constrained. In contrast, a dust dissolution test showed that the release of PO_4^{3-} from dust was found to be constant after 6 h. Phosphate uptake rates could be calculated from the initial and final concentration of PO_4^{3-} and the amount of PO_4^{3-} released from dust particles (Fig. 4b). Phosphate assimilation was significantly enhanced for the three dust addition treatments relative to the control (one-way ANOVA, $p < 0.05$, Fig. 4b). While dust addition stimulated N_2 fixation of *Trichodesmium*, the effect was not statistically significant compared to the control (one-way ANOVA, $p > 0.05$, Fig. 4c). Carbon fixation of *Trichodesmium* increased significantly in response to dust concentrations (one-way ANOVA, $p < 0.05$, Fig. 4d).

3.2. Field incubation experiments

The physical and chemical characteristics of the surface seawater along the cruise transect in the Bay of Biscay and along the Iberian Margin showed a clear north to south pattern, with a transition from cold and nutrient-rich waters (Bay of Biscay) into warm and nutrient-depleted oligotrophic waters (Iberian Margin) (Table 2).

Vertical profiles of N_2 fixation rates determined at PAR levels of 54%, 13%, 3% and 0.2% of the incident irradiance in the Bay of Biscay and along the Iberian Margin show a higher N_2 fixation activity in surface waters compared to depth (Fig. A.1). Studies on the natural distribution of cyanobacteria are often limited to surface waters. Since the highest N_2 fixation rates measured in our study area were restricted to surface waters, the effect of dissolved Fe on oceanic N_2 fixation rates was investigated by incubating surface seawater samples containing natural phytoplankton assemblages. We compare the N_2 fixation rates with and without Fe addition in Fig. 5. At Sts 3 and 5, natural N_2 fixation rates were not measurable, while after Fe addition rates went up to around 100 nmol L^{-1} d^{-1}. At Sts 7, 9 and 13, N_2 fixation rates were tremendously enhanced after Fe addition, increasing 80, 20 and

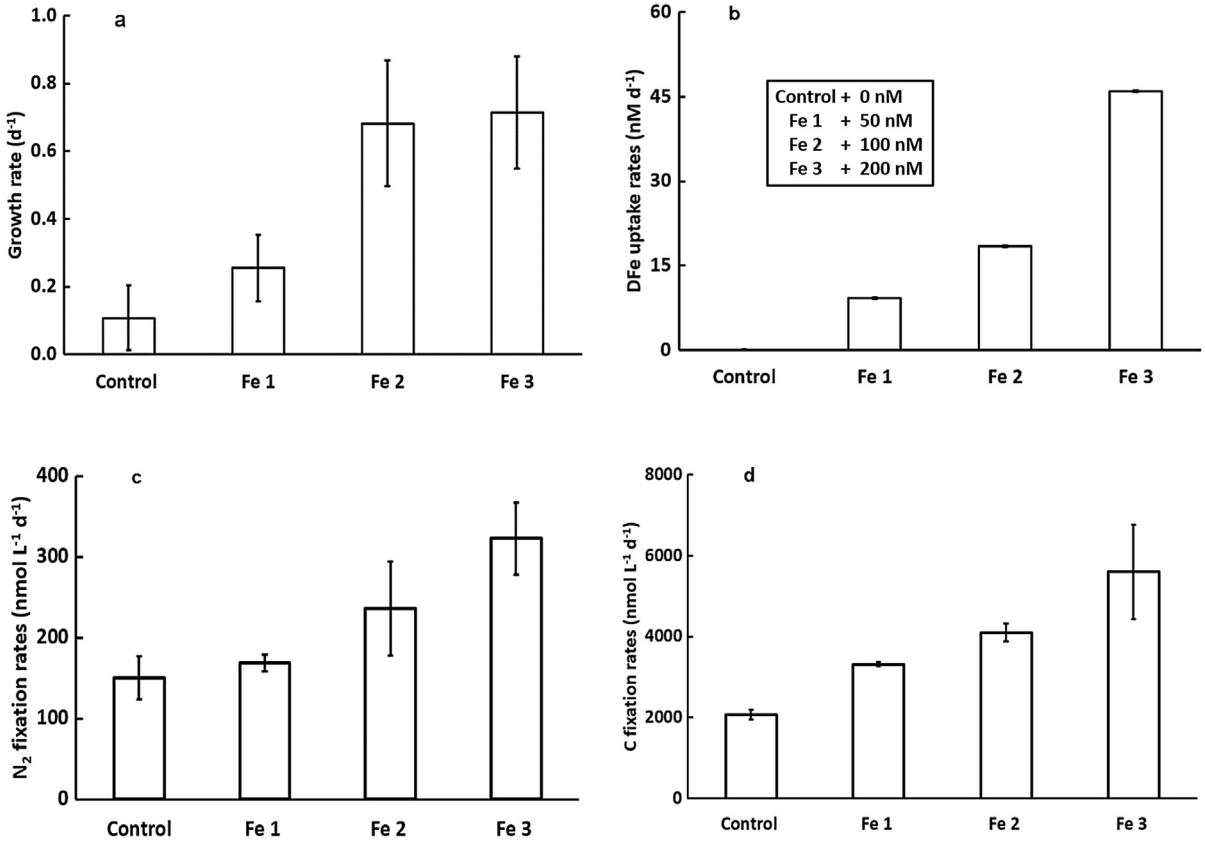

Fig. 2. (a) Growth rates, (b) DFe uptake rates, and (c) N_2 and (d) C fixation rates of *Trichodesmium* IMS101 for Control (no Fe addition) and three +Fe (50, 100 and 200 nM) addition treatments during 24 h incubations.

40 times, respectively. With Fe addition, the N_2 fixation rates at St 13 even increased up to around 2000 nmol L^{-1} d^{-1}.

The spatial variation in the response of N_2 fixation activities could be explained by a spatial variability in the abundance of N_2 fixers. Through molecular biology techniques based on the applied protocol, 103 *nifH* sequences were recovered exclusively in surface seawater samples (5 m) at Sts 11 and 13, but were below detection limit at other stations and depths (Fonseca-Batista et al., 2018). The recovered *nifH* sequences belong mostly to *Candidatus Atelocyanobacterium thalassa* (heterotrophic diazotrophic cyanobacteria, UCYN-A1), but also to other heterotrophic bacteria such as Bacteriodetes, Firmicutes and Proteobacteria (Fonseca-Batista et al., 2018). At St 11, all *nifH* sequences (41 of 103) were found to be UCYN-A1. However, at St 13 diverse N_2 fixers were observed, with 45.2% of the sequences affiliated to UCYN-A1, Bacteriodetes 25.8%, Firmicutes 19.3% and Proteobacteria 9.7% (Fonseca-Batista et al., 2018).

3.3. Semi-continuous dilution growth experiments

Our results revealed that while growth rates of *Trichodesmium* IMS101 significantly increased with increasing pCO_2 (two-way ANOVA, $p < 0.05$, Fig. 6a), increasing temperature had no significant effect (two-way ANOVA, $p > 0.05$, Fig. 6a). Growth rates at an elevated pCO_2 level (800 µatm) were significantly higher than those at the present-day pCO_2 level (400 µatm) regardless of the temperatures (two-way ANOVA, $p < 0.05$, Fig. 6a). Similarly, cell-number normalized POC and PN production rates, N_2 fixation and C fixation rates of *Trichodesmium* IMS101 also showed a significant increase in response to increasing pCO_2 (two-way ANOVA, $p < 0.05$, Fig. 6b–e). But no significant differences were observed with changes in temperature (two-way ANOVA, $p > 0.05$, Fig. 6b–e). With increasing CO_2 concentrations, *Trichodesmium* IMS101 correspondingly raised both their cell-number normalised P and DFe uptake rates to meet the growth requirements (Fig. 6f, g). The difference was that the increased cell-number normalised P uptake rates were statistically significant (two-way ANOVA, $p < 0.05$, Fig. 6f), while DFe uptake rates were found to be highly variable and not statistically significant (two-way ANOVA, $p > 0.05$, Fig. 6g). Again, temperature did not significantly affect the cell-number normalized P nor the DFe uptake rates (Fig. 6f, g) (two-way ANOVA, $p > 0.05$). This observation is consistent with the results obtained for overall growth rates (Fig. 6a), cellular POC and PN production rates (Fig. 6b, c), as well as N_2 and C fixation rates (Fig. 6d, e).

4. Discussion

4.1. Nutrient control of N_2 fixation

4.1.1. Fe limitation

Growth rate and N_2 and C fixation of *Trichodesmium* were stimulated with DFe additions at 50, 100 and 200 nM (Fig. 2a, c, d). The effects increased with increasing Fe addition, although some of the responses were not statistically significant (One-way ANOVA, $p > 0.05$). The finding that Fe availability controls the growth and N_2 fixation activity of *Trichodesmium* IMS101 is consistent with previous investigations based on laboratory cultures and natural populations (Berman-Frank et al., 2001; Wu et al., 2003; Mills et al., 2004; Moore et al., 2009; Boatman et al., 2018). Note that while the concentrations of DFe in our culture media (50–200 nM) are appropriate for the dense culture conditions, they are 2–3 orders of magnitude higher compared to other culture experiments (e.g., DFe < 10 nM; Boatman et al., 2018) and natural seawaters (DFe < 1.2 nM; Moore et al., 2009). Thus the DFe levels in our culture experiments are not suitable for identifying any true limitation by DFe. For *Trichodesmium* IMS101 cultured under different Fe regimes, Fe limitation resulted in a significant decline of

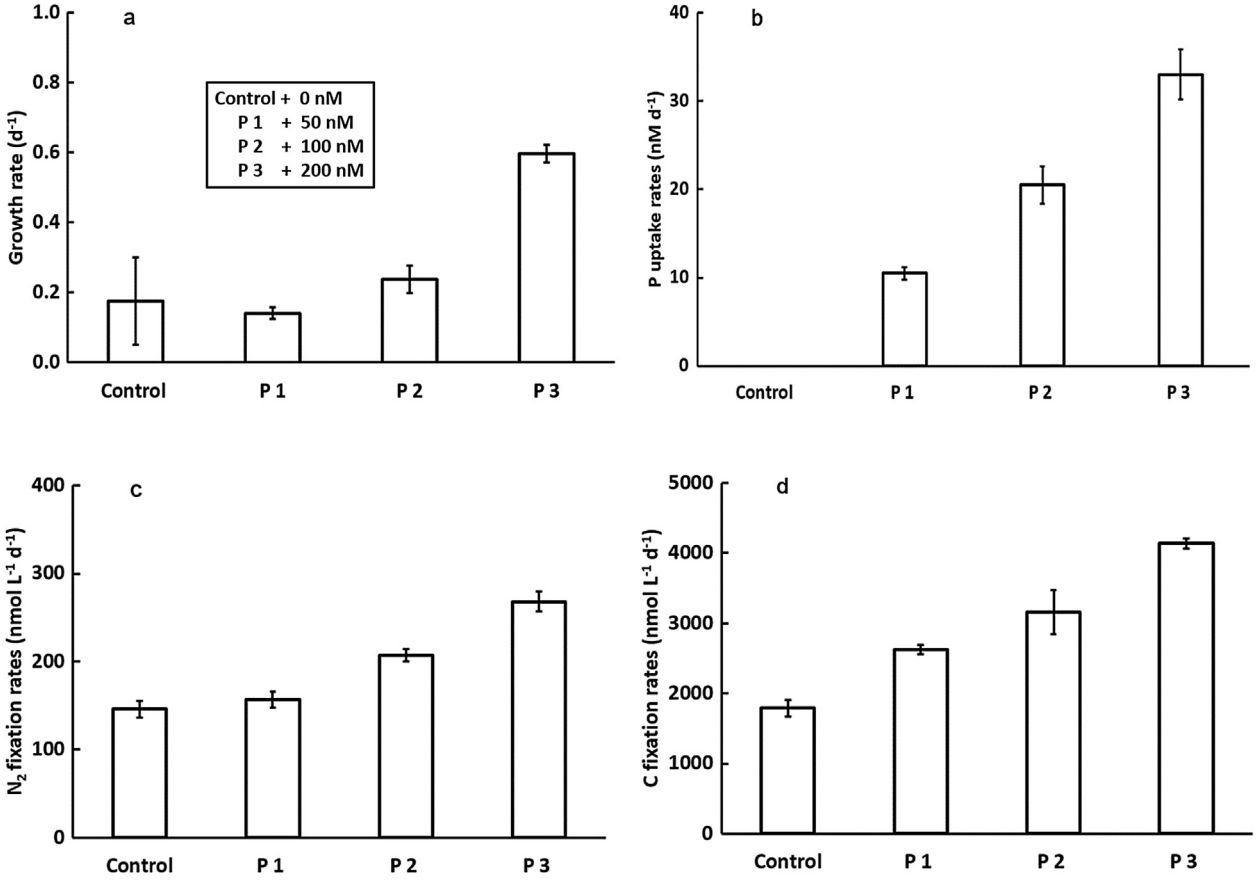

Fig. 3. (a) Growth rates, (b) P uptake rates, and (c) N_2 and (d) C fixation rates of *Trichodesmium* IMS-101 for Control (no P addition) and three +P (50, 100 and 200 nM) addition treatments during 24 h incubations.

specific growth rate, photosynthesis and N_2 fixation rate (Boatman et al., 2018). Moore et al. (2009) observed a positive correlation between dissolved Fe concentration and N_2 fixation rates for surface waters along a 10,000 km north–south section in the Atlantic Ocean and concluded that inter-basin differences in N_2 fixation are controlled by Fe supply. Based on a model output Berman-Frank et al. (2001) concluded that N_2 fixation by *Trichodesmium* could be essentially Fe-limited in 75% of the world oceans.

In our laboratory experiments, there was no measurable Fe uptake for the Fe-limiting treatment (control) (Fig. 2b). However, DFe uptake rates increased with dissolved Fe additions suggesting a lower Fe requirement under Fe-limited conditions and a luxury uptake and storage under Fe-replete conditions (Fig. 2b). In addition, based on cell-number normalised Fe and C uptake data (not shown), calculated Fe quotas in our study were very high with Fe:C values ranging from 2000 to 8000 μmol:mol, due to high Fe concentrations, which were about 1–2 orders of magnitude higher compared to other published values (e.g., Fe:C ratio ranging between 13.5 and 923 μmol:mol; Berman-Frank et al., 2001, 2007; Kustka et al., 2003a, 2003b). In general, higher Fe:C uptake ratios were observed for cultures grown under high Fe concentrations. Thus, with Fe concentrations 2–3 orders of magnitude higher, we observed Fe:C uptake ratios that are 1–2 orders of magnitude higher as compared to values found in the literature suggesting indeed the existence of luxury Fe uptake mechanism. Luxury Fe uptake and storage is widely considered as one of the most important adaptations developed by *Trichodesmium* in the oligotrophic ocean (Kustka et al., 2003b; Chen et al., 2011). Chappell and Webb (2010) report that this behavior involves a re-regulation of Fe-containing proteins responsible for photosynthesis and N_2 fixation under different DFe concentrations. Furthermore, although *Trichodesmium* has no genetic potential to produce siderophores, they could possibly constitute a symbiotic relationship with heterotrophic bacteria, thereby potentially increasing the Fe bioavailability through the siderophores produced by these associated bacteria (Achilles et al., 2003; Rouco et al, 2016; Lee et al., 2017). It has been shown that *Trichodesmium* can further increase the uptake rates of siderophore-bound Fe by forming radial 'puff' colonies (Achilles et al., 2003).

4.1.2. P limitation

For cultures grown under P-limiting conditions we measured an increase in growth, C and N_2 fixation rates of *Trichodesmium* IMS101 with PO_4^{3-} fertilization at 50, 100 and 200 nM (Fig. 3a, c, d), and these responses became enhanced with increasing P additions. Yet some of the responses were not statistically significant (one-way ANOVA, $p > 0.05$). In accordance with previous research, our P addition experiments verified the effect of P availability on both *Trichodesmium* growth and N_2 fixation. In the Southwest Pacific Ocean, *Trichodesmium* spp. growth and distribution appeared to be controlled by seasonal variations in P availability (Moutin et al., 2005). Dust bound-Fe stimulation of N_2 fixation in the North Atlantic Ocean could in turn lead to P limitation, with PO_4^{3-} concentrations as low as 1 nM (Wu et al., 2000). However, P content in crustal materials is very low, ranging between 0.1% and 0.2% by weight (Duce et al., 1991). The desert dust used in our study comprises 0.19% of P (Li et al., 2017). The low P content and low P:Fe ratio from desert dust release observed in our study indicate that while dust deposition may be an important source of Fe for N_2 fixers, the input of P from dust is less important and would have a smaller impact on marine productivity, in agreement with other studies (Duarte et al., 2006; Krishnamurthy et al., 2010).

No P uptake was observed for the P-limited control experiment (Fig. 3b). In contrast, P uptake rates increased in response to increasing P additions (Fig. 3b). This suggests that *Trichodesmium* has the capability

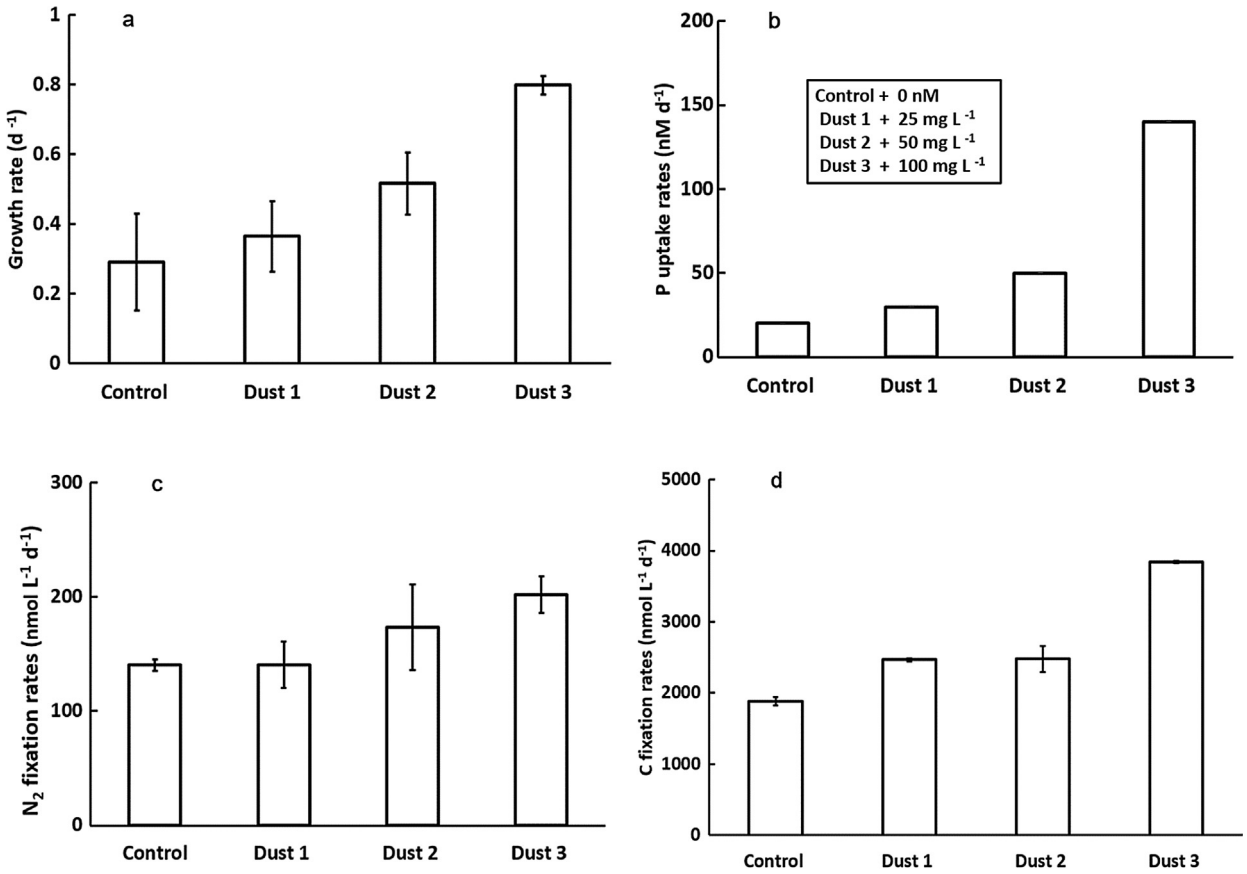

Fig. 4. (a) Growth rates, (b) P uptake rates, and (c) N_2 and (d) C fixation rates of *Trichodesmium* IMS-101 for Control (no dust addition) and three + Dust (25, 50 and 100 mg L^{-1}) addition treatments during 24 h incubations.

Table 2
Initial environmental conditions of the surface seawater in the Bay of Biscay and along the Iberian Margin.

	St 3	St 5	St 7	St 9	St 11	St 13
Temperature (°C)	14.0	14.1	14.0	16.0	16.9	17.2
Salinity	35.6	35.6	35.52	35.54	35.54	35.68
Chl-a (μg L^{-1})	1.05	0.15	1.17	0.17	0.17	0.10
NO_3^- (μM)	0.27	0.48	0.10	0.09	0.08	0.02
PO_4^{3-} (μM)	0.05	0.02	0.03	0.01	0.01	0.01
DSi (μM)	0.44	0.15	0.13	0.31	0.31	0.23
DFe (nM)	15	2	10	20	20	20
POC (μM)	13.43	7.01	14.12	5.16	5.36	4.88
PN (μM)	1.98	0.93	1.87	0.65	0.47	0.45

to regulate its P requirement in response to shifts in ambient PO_4^{3-} concentrations, for instance, via a reduction of the P cell quota under P deficiency, the so-called "P-sparing effect" (Karl et al., 2002). For the control treatment without P addition we observed some N_2 fixation activity while no P uptake was detected, possibly indicating that other forms of P were being used (Fig. 3b, c). Indeed, *Trichodesmium* spp. have genes coding for dissolved organic P (DOP) utilization enzymes (e.g. alkaline phosphatase and C-P lyase), making DOP accessible in the form of both phosphomonoesters and phosphonates, which may be less bioavailable to the general microbial population (Sohm et al., 2008; Orchard et al., 2009; 2010). The utilization of DOP by diazotrophs has been suggested to support N_2 fixation in the North Atlantic (Coles and Hood, 2007). The capacity to maximize P uptake via the uptake of both inorganic and organic P species, in particular, to hydrolyse semi-labile DOP and access the more refractory phosphonates may give *Trichodesmium* spp. or N_2 fixers a competitive advantage over other organisms in oligotrophic P-depleted environments (Landolfi et al., 2015).

4.1.3. Dust deposition

In the dust addition experiments, *Trichodesmium* IMS101 was kept under Fe- and P- depleted conditions. For the three different addition levels of 25, 50 and 100 mg L^{-1}, desert dust particles stimulated growth, and C and N_2 fixation rates of *Trichodesmium* IMS101 compared to cultures without dust addition (Fig. 4a, c, d). Furthermore, the above-mentioned effects increased with increasing amount of dust added. There is accumulating evidence suggesting that the delivery of Fe and P to the oceans through dust deposition may relieve the Fe and P co-limitation of N_2 fixation and may ultimately constrain the rate of N_2 fixation on the global ocean scale (Mills et al., 2004; Ridame et al., 2011; Langlois et al., 2012). Our observation that growth and N_2 fixation of *Trichodesmium* IMS101 are stimulated by the addition of Kubuqi desert dust is consistent with this suggestion. By supplying both Fe and P, Saharan dust additions presumably stimulated N_2 fixation in the tropical North Atlantic, a region rich in diazotrophs and strongly impacted by Saharan dust input (Mills et al., 2004). In addition, Moore et al. (2009) highlighted the correlation of *Trichodesmium* distribution, peak N_2 fixation rates and Saharan dust deposition on a transect from the North Atlantic through the South Atlantic Ocean. However, as also mentioned above, while dust deposition can provide sufficient Fe, it represents only a minor source of P relative to the large P requirements of N_2 fixers. Interestingly, Garcia et al. (2015) observed higher growth and N_2 fixation rates of *Trichodesmium* under Fe deficient conditions compared to Fe replete but P-deplete conditions.

Under DFe and P co-limiting conditions, P uptake rates of *Trichodesmium* IMS101 increased in concert with dust concentrations, suggesting a possible P luxury uptake mechanism (Fig. 4b). Even though it was difficult to quantify the DFe uptake rates, the concurrent activation of Fe and P luxury uptake mechanisms seemed likely since the release of Fe from the dust particles varied widely. However, the uptake processes and mechanisms associated with dust acquisition by

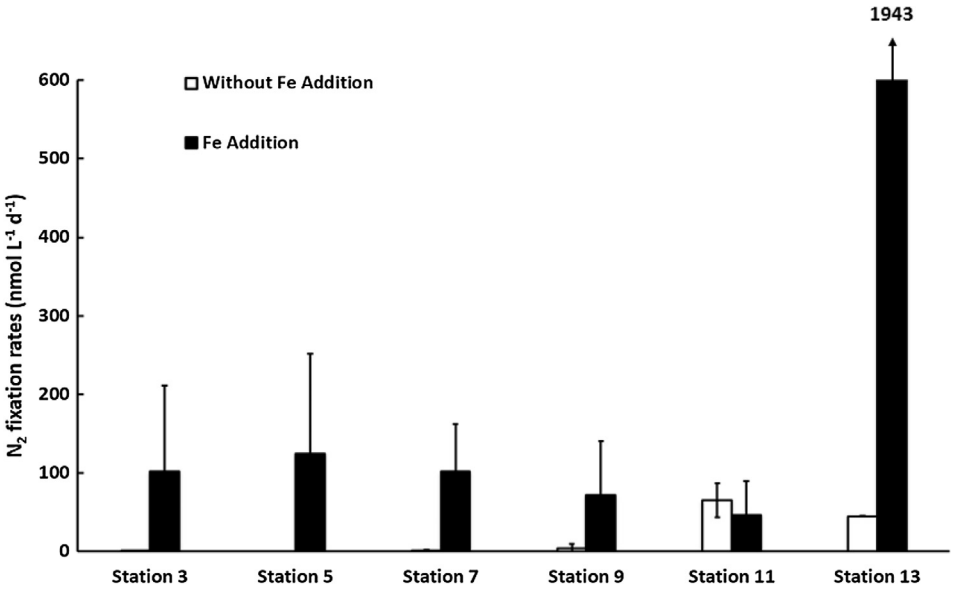

Fig. 5. N_2 fixation rates with and without dissolved Fe addition (100 nM) in surface seawater sampled at 5 m in the Bay of Biscay and along the Iberian Margin.

Trichodesmium are poorly understood. Phosphate is basically thought to be immediately bioavailable after dust dissolution. The difficulty therefore lies in how *Trichodesmium* is able to use aeolian dust as an additional source of Fe or make the Fe released from dust bioavailable. Several effective pathways such as mobilization by organic ligands, photoreduction and bio-reduction may be involved in the *Trichodesmium*-mediated dissolution of iron minerals (LaRoche and Breitbarth, 2005; Rubin et al., 2011; Sohm et al., 2011). The active role of *Trichodesmium* colonies in collecting, storing and processing dust particles has been reported for natural populations and laboratory cultures of *Trichodesmium* (Rueter et al., 1990, 1992; Rubin et al., 2011). *Trichodesmium* could actively accelerate the rate of Fe dissolution from ferrihydrite and dust minerals and enhance Fe bioavailability through cell surface processes (Rubin et al., 2011).

4.1.4. Role of Fe: a field study

At most stations N_2 fixation rates in oligotrophic surface seawaters were strongly stimulated by the addition of dissolved Fe, demonstrating the limitation of N_2 fixation by Fe in the Bay of Biscay and along the Iberian Margin (Fig. 5). However, the intensity of stimulation varied between sites, possibly because of variable initial nutrient conditions (such as NO_3^-, PO_4^{3-} and DFe) of the surface seawater used for the incubation experiments.

In the studied region where sea surface temperature (SST) ranged from 13 to 18 °C, no *Trichodesmium* filaments were observed and the diazotrophic community was dominated by unicellular cyanobacteria symbiont (prymnesiophyte-UCYN-A1) and heterotrophic diazotrophs (Fonseca-Batista et al., 2018). These smaller diazotrophs have been detected in colder (SST < 15 °C) North Atlantic waters as far north as 50°N (Langlois et al., 2008; Rees et al., 2009; Agawin et al., 2014). Many environmental factors can affect N_2 fixation, but P and Fe are thought to be the most important co-limiting factors in most regions. Along a North-South Atlantic Ocean transect between 37°N and 35°S, Moore et al. (2009) found that N_2 fixation was correlated positively with dissolved Fe concentrations and negatively with dissolved P concentrations. This observation indicates that N_2 fixation in the Atlantic Ocean is mainly controlled by Fe availability rather than P, although P was usually thought to be the more severely limiting nutrient. In the subtropical eastern North Atlantic Ocean, Krupke et al. (2015) investigated the effect of nutrients (P, Fe and dust) on N_2 fixation by a prymnesiophyte-UCYN-A1 association. They found that N_2 fixation by UCYN-A cells was significantly stimulated by the addition of DFe and Saharan dust (which served as a source of both Fe and P), but not by the addition of P only, implying that the ultimate limiting factor for N_2 fixation of UCYN-A could be Fe. Similar results were obtained in the present study. The PO_4^{3-} concentrations in all surface samples were extremely low, below 0.05 μM, but N_2 fixation rates were still enhanced with DFe addition under such low P conditions, suggesting that there was an additional P source that could have satisfied the P requirement for biological N_2 fixation (Table 2, Fig. 5). It has been shown that N_2-fixers can utilize the dissolved organic phosphorus (DOP) pool as an additional source of P in most oligotrophic environments (Landolfi et al., 2015). Therefore, indeed DFe could be the ultimate factor limiting N_2 fixation by these smaller diazotrophs in low temperature seawater depleted in P.

4.2. Environment control of N_2 fixation

4.2.1. Ocean acidification

Our preliminary findings suggest that growth rates (Fig. 6a), POC and PN production rates (Fig. 6b, c), and N_2 and C fixation rates (Fig. 6d, e) of *Trichodesmium* IMS101 were all significantly enhanced by elevated pCO_2 under both low and high temperature conditions. The impact of enhanced pCO_2 on *Trichodesmium* IMS101 has been actively investigated by others and the CO_2 stimulation observed in our study is consistent with previous investigations on *Trichodesmium*. This indicates that increased pCO_2 could support higher growth rates and biomass production as well as stimulate rates of N_2 and C fixation (Barcelos e Ramos et al., 2007; Hutchins et al., 2007; Levitan et al., 2007, 2010; Kranz et al., 2009, 2010). Nonetheless, the magnitude of these pCO_2-dependent effects differed significantly between studies. For instance, the enhancement of N_2 fixation and C fixation ranged from 35% to 200% and from 15% to 100%, respectively, between present-day pCO_2 and those predicted for the year 2100 (750–1000 μatm) (Barcelos e Ramos et al., 2007; Hutchins et al., 2007; Levitan et al., 2007, 2010; Kranz et al., 2009, 2010). The observed increase in growth and N_2 fixation rate of *Trichodesmium* in response to elevated pCO_2 could result from a down-regulation of high-energy demanding Carbon Concentrating Mechanisms (CCM) at high CO_2 concentrations and a reallocation of energy to other cellular processes, such as N_2 fixation (Levitan et al., 2007, 2010; Kranz et al., 2009, 2010).

However, the responses of *Trichodesmium* IMS101 to elevated CO_2 are not consistent between studies due to differences in P and DFe concentrations of the culture media used. In our Fe-replete and P- replete cultures, we observed a considerable increase of both cell-number normalized P (Fig. 6f) and Fe (Fig. 6g) uptake rates for high pCO_2

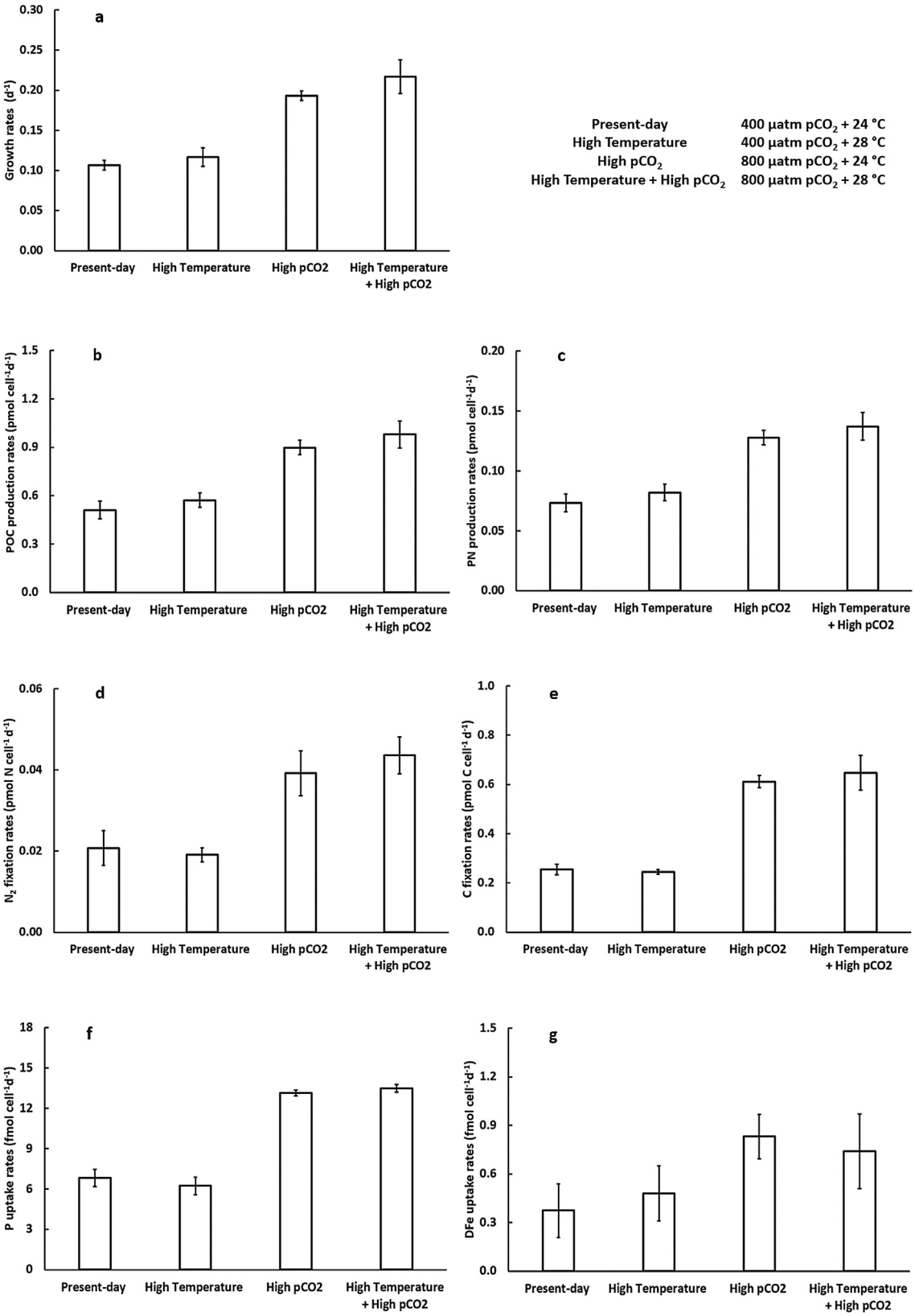

Fig. 6. (a) Growth rates and cell-number normalised (b) POC and (c) PN production rates, (d) N_2 and (e) C fixation rates, and (f) P and (g) DFe uptake rates of *Trichodesmium* IMS 101 acclimated to different pCO_2 and temperature conditions during 24 h incubations. The various treatments correspond to Present-day (400 µatm pCO_2 and 24 °C), High Temperature (400 µatm pCO_2 and 28 °C), High pCO_2 (800 µatm pCO_2 and 24 °C), and High Temperature + High pCO_2 (800 µatm pCO_2 and 28 °C) conditions.

treatments. Shi et al. (2012) on the contrary, reported that increasing pCO_2 and decreasing pH led to a decrease in the rates of N_2 fixation and growth in *Trichodesmium* and a reduction in Fe uptake rates under low-Fe conditions. These results indicate that the positive effects of CO_2 enrichment on *Trichodesmium* growth and N_2 fixation may be reduced by Fe limitation. Moreover, by examining the combined effects of P availability and adaptation to increasing CO_2, Hutchins et al. (2007) observed that the effect of pCO_2 on *Trichodesmium* was independent of P concentrations since the relative impact of elevated pCO_2 was similar under P-limiting and P-replete conditions.

Complicating matters further, Hong et al. (2017) reported that CO_2 stimulation on N_2 fixation activity of *Trichodesmium* might be artefactual due to ammonia and/or copper contamination of the artificial seawater YBCII medium and the positive effect of high CO_2 was reversed when using low ammonia and high EDTA uncontaminated medium. However, Hutchins et al. (2017) questioned these conclusions and stated that the high N_2 fixation activities observed in the contaminated YBCII medium could not be well explained by this toxic contamination hypothesis. In addition, several previous studies on *Trichodesmium* found significant positive impact of high CO_2 on growth and N_2 fixation in the same uncontaminated medium (Hutchins et al., 2017, and references therein).

Moreover, different N_2 fixers may respond differently to ocean acidification (Hutchins et al., 2013; Eichner et al., 2014). For three diazotroph species (a UCYN-C and two heterocystous species) which were exposed to rising pCO_2 (980 µatm), Eichner et al. (2014) observed enhanced, reduced as well as unaffected growth and N_2 fixation activity. The variable response patterns may be related to the functional diversity in modes of N_2 fixation as well as to differences in cellular energy limitation within diazotroph groups and species (Eichner et al., 2014). For instance, as described above, *Trichodesmium* spp. can down-regulate the high energy demanding CCM and therefore free energy to up-regulate N_2 fixation in response to increasing CO_2 availability. UCYN-A lacks a number of major metabolic pathways including C fixation and consequently may be unaffected by changes in CO_2 availability (Tripp et al., 2010). However, UCYN-A cells are compulsorily associated with the eukaryotic prymnesiophyte which cannot utilise N_2 as an N source and can receive fixed N from UCYN-A in exchange for transferring fixed C to the latter (Thompson et al., 2012; Krupke et al., 2013). Although UYCN-A itself may be insensitive to changing pCO_2, the prymnesiophyte partner may respond to different CO_2 conditions and thus indirectly affect N_2 fixation activity of UYCN-A (Krupke et al., 2015). In view of the wide variety in responses to CO_2 among N_2 fixers, future rising atmospheric CO_2 concentrations and OA could lead to changes in their spatial distribution and community composition.

4.2.2. Ocean warming

Trichodesmium IMS101 strains were kept and acclimated at 24 °C and 28 °C. Contrasting with the responses to increasing pCO_2 level, our preliminary results indicate that growth rates (Fig. 6a), POC and PN production rates (Fig. 6b, c), and N_2 and C fixation rates (Fig. 6d, e) of *Trichodesmium* IMS101 were not significantly influenced by a 4 °C temperature increase under both low and high pCO_2 conditions. Accordingly, no significant difference in cell-number normalized Fe (Fig. 6f) and P (Fig. 6g) uptake rates was found with increasing temperature. The insignificant impact of temperature was in good agreement with the work previously reported for *Trichodesmium* IMS101 by Hutchins et al. (2007), who observed that growth rates, photosynthesis and N_2 fixation were affected only minimally by a 4 °C change from 25 °C to 29 °C.

Temperature has long been considered as a major factor controlling global abundance and distribution of *Trichodesmium* and their N_2 fixation rates. In biogeochemical general circulation models, *Trichodesmium* is generally assumed to be restricted to oligotrophic waters with a temperature range from 20 °C to 30 °C (Breitbarth et al., 2007). For *Trichodesmium erythraeum*, the minimum temperature limit (T_{min}), the optimum temperature (T_{opt}) and maximum temperature limit (T_{max}) range between 19–20 °C, 26–27 °C and 32–34 °C, respectively (Breitbarth et al., 2007; Fu et al., 2014; Boatman et al., 2017). Thus, it is not surprising that there was no significant change when *Trichodesmium* was grown at 24 °C (approaching T_{opt}) and 28 °C (slightly above T_{opt}) in our cultures. However, choosing a different range of temperatures probably would lead to different results. For instance, selecting temperature increments between 28 °C and 32 °C, or 22 °C and 26 °C might have yielded a very different conclusion, with either inhibiting or stimulating effects. Thus, it should be noted that the effect of temperature on *Trichodesmium* growth is complex and not linear, depending on the selected temperature range.

With ongoing surface ocean warming and increasing stratification, oligotrophic subtropical conditions are expected to extend to higher latitudes. Correspondingly, *Trichodesmium* spp. also has the potential to shift poleward, leading to an 11% areal increase of its potential spatial distribution (Breitbarth et al., 2007). This future expansion of *Trichodesmium* distribution in the subtropical gyres could subsequently change the magnitude of global N_2 fixation, with a predicted increase of N_2 fixation by 27% (Boyd and Doney, 2002). However, the negative skewness of a thermal tolerance curve indicates that a potential sudden sharp decline in *Trichodesmium* growth and N_2 fixation could occur if the ocean continues to warm with a 3–4 °C increase above the T_{opt} (Boatman et al., 2017). In a future warmer ocean large tropical and subtropical regions are projected to approach or reach beyond the optimal temperature range of *Trichodesmium* (Fu et al., 2014). As a consequence, a narrowing of the *Trichodesmium* distribution area in these tropical and subtropical waters may be offset by the enlarged coverage and enhanced marine N_2 fixation at higher latitudes.

5. Conclusions

The recognition of the importance of N_2 fixation in the global cycling of C and N has recently inspired studies investigating the major factors controlling N_2 fixation and the growth of diazotrophs, particularly in the case of the filamentous diazotroph *Trichodesmium*. Among these factors, the impacts of nutrients (Fe and P) and dust deposition on *Trichodesmium* distribution and N_2 fixation have been confirmed by a large number of field and laboratory studies. For the laboratory bioassays (+Fe, +P, +Dust) performed on *Trichodesmium* IMS101 we found that both Fe and P additions stimulate the growth and the N_2 fixation rates. Additions of desert dust could relieve the Fe and P co-limitation of N_2 fixation, suggesting the importance of dust inputs in marine N_2 fixation potentially in oligotrophic oceans. A number of novel nutrient acquisition and utilization strategies have been discovered for *Trichodesmium* to adapt nutrient-limited condition, such as luxury Fe uptake and storage, P-sparing effect and colony formation. In addition, based on the *nifH* expression analysis, stable isotope and Fe enrichment incubations in the Bay of Biscay and along the Iberian Margin during the Belgica cruise in May 2014, we provide evidence for the significance of Fe availability for N_2 fixation in the temperate Northeast Atlantic Ocean. N_2 fixation activity was remarkably stimulated through the addition of dissolved Fe, demonstrating that local diazotrophs thrived under Fe limiting conditions. Since N_2-fixers are assumed to be capable of using the DOP pool as an additional P source in most oligotrophic environments, dissolved Fe concentration could be the ultimate limiting factor for N_2 fixation.

In view of the ongoing increase in CO_2 concentrations and temperature it is crucial to assess, understand and predict future responses to ocean acidification and warming of ecologically important N_2-fixing cyanobacteria *Trichodesmium* spp. In our semi-continuous batch cultures of *Trichodesmium* IMS101, growth rates, POC and PN production rates, and N_2 and C fixation rates were all significantly enhanced by elevated pCO_2 indicating that higher pCO_2 and therefore ocean acidification may be beneficial for *Trichodesmium* growth and N_2 fixation. However, we propose that Fe or P limitation in oligotrophic seawaters may offset the stimulation by ocean acidification. In contrast, growth and N_2 and C fixation rates of *Trichodesmium* IMS101 were not significantly influenced by a 4 °C increase going from 24 to 28 °C. However, ocean warming is predicted to shift the geographical distribution of *Trichodesmium* species

toward higher latitudes, extending its niche to subtropical oceanic regions and reducing its coverage in the tropical ocean.

In order to better understand the environmental and nutrient controls of marine nitrogen fixation, more extensive studies about the following aspects are required:

(1) Combined or interactive effects of multiple environmental variables and stressors, in combination of CO_2, temperature, dust deposition, light and nutrient availability, *etc.* (Garcia et al., 2013, 2015; Boatman et al., 2018). The future ocean is predicted to face a multitude of changes. Joint effects of changes in two or more control factors may lead to additive, synergistic or antagonistic effects, resulting in significant modifications in marine biogeochemical cycles as well as transitions in oceanic microbial communities and even ecosystem structure.
(2) Multifarious taxon-, species- and strain-specific responses to climate change (Hutchins et al., 2013; Eichner et al., 2014). The findings on various genera, species and strains should help us to predict evolutionary changes within species and how the community compositions might change in the future ocean.
(3) Prospective long-term physiological acclimatization or evolutionary adaptation in order to keep pace with the rate of climate change (Vogt, 2015; Hutchins et al., 2015). Generally, short-term culture experiments do not inform about the adaptive processes. The observed responses may therefore overestimate or underestimate the long-term sensitivity of natural populations to climate change.

6. Declaration of interest

The authors have declared that no competing interests exist.

7. CRediT authorship contribution statement

Xuefeng Li: Methodology, experimental design, analysis, data processing, original manuscript writing; **Debany Fonseca-Batista:** Methodology, experimental design, analysis, data processing, manuscript review; **Nathalie Roevros:** Analysis, resources; **Frank Dehairs:** Methodology, resources, supervision, manuscript review; **Lei Chou:** Methodology, resources, supervision, manuscript review. All authors have approved the final article.

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Appendix A

See Fig. A.1.

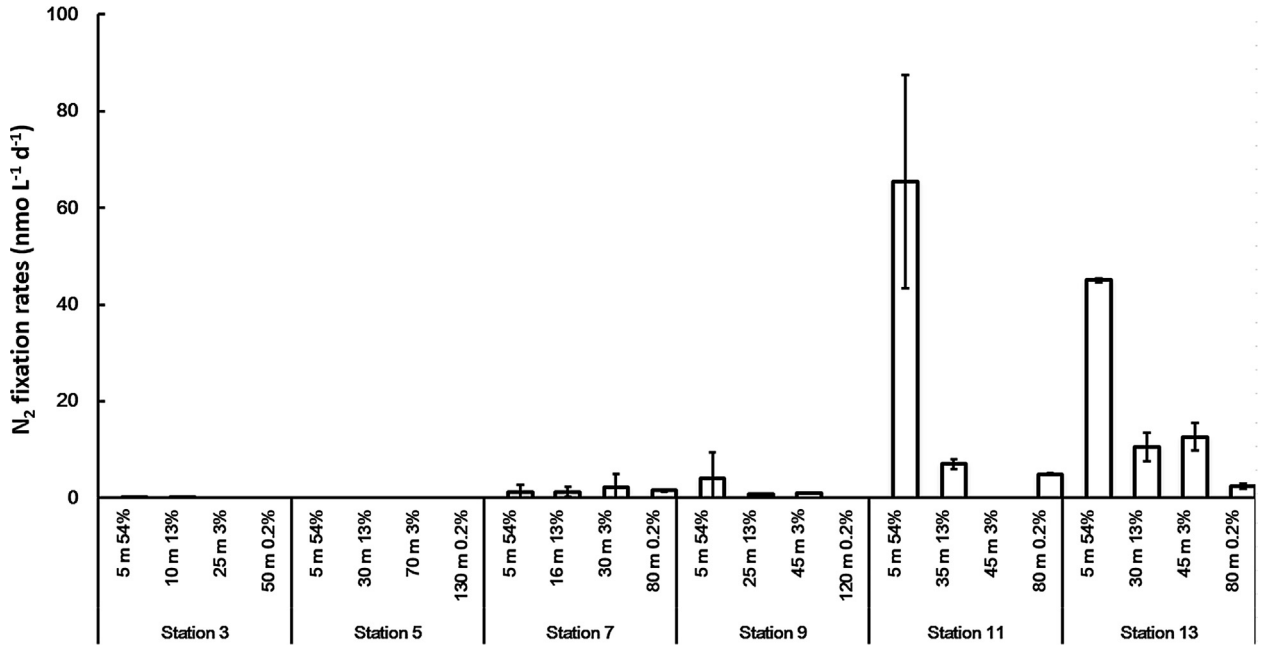

Fig. A.1. Natural N_2 fixation rates at four different levels of light transmission (54%, 13%, 3% and 0.2%) in the Bay of Biscay and along the Iberian Margin.

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